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Lack of requirement for Presenilin1 in Notch1 signaling

B.E. Berechid*, G. Thinakaran†, P.C. Wong‡, S.S. Sisodia† and J.S. Nye*§

Studies in invertebrates have indicated a functional requirement for presenilin (PS) genes in the Notch pathway [1–5]. One model of Notch signal transduction suggests that proteolysis releases an activated Notch fragment that migrates to the nucleus and regulates gene transcription in concert with CBF1/Su(H)/lag1 (CSL) proteins [6–9]. Recent studies suggest that PS genes control the proteolysis and nuclear access of the Notch intracellular domain [3,4,10,11], offering a basis for the functional interaction of PS and Notch genes [12]. Here, we report that Notch1 signaling elicited by the ligand Delta1 was quantitatively unchanged in PS1-deficient primary embryonic fibroblasts (PEFs). Notch1 signals were measured by both the activation of the *hairy/enhancer of split* (*HES1*) promoter and by the antagonism of MyoD-induced muscle creatine kinase (*MCK*) promoter activity. A membrane-tethered ligand-independent Notch1 construct also showed full efficacy in both assays, despite its presumed requirement for cleavage. Although signaling through Notch1 persisted in PS1-deficient cells, we found a marked reduction in the appearance of a complex of a cleaved, intracellular Notch fragment (NICD) and a CSL protein, as previously reported [6,10]. These studies reveal that PS1 is not required for ligand-dependent Notch signaling, and that PS1 and PS2 may be redundant. Our data also suggest that the identified NICD fragment may not be necessary for Notch signal transduction [9].

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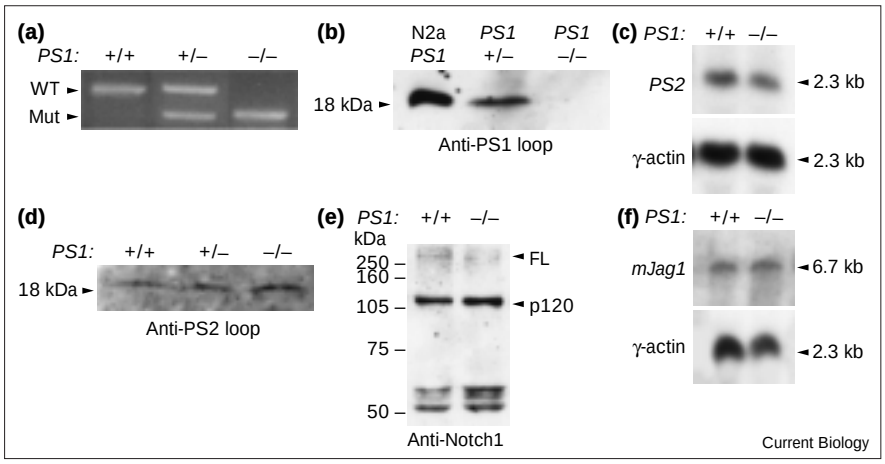
Results and discussion

In order to measure the effect of PS1 deficiency on ligand-dependent and ligand-independent Notch signaling, we established PEFs from *PS1* heterozygous intercrosses [13] (Figure 1). *PS1*$^{-/-}$ PEFs did not express *PS1* but did express *PS2* mRNA and a mature, carboxy-terminal fragment of PS2 protein at similar levels to those seen in *PS1*$^{+/+}$ cultures. PEFs expressed Notch1 protein, as detected by an antibody directed to its intracellular domain. Neither the quantity of the 120 kDa intracellular domain, nor the small amount of full-length protein (250 kDa) was significantly altered by the absence of PS1 in these cells. Additionally, the expression of *Jagged1* mRNA, which encodes a ligand of Notch proteins, was not altered in *PS1*-null cells compared to control (Figure 1).

In mammalian cells, Notch signals induce the expression of the *HES1* gene and can antagonize MyoD-mediated activation of muscle-specific promoters [14,15]. These effects are thought to be accomplished by an association of the intracellular domain of Notch with a CSL protein [9], although a CSL-independent pathway may also have a role in MyoD antagonism [16]. To test the role of PS1 in Delta1-dependent *HES1* activation [17], *PS1*$^{+/+}$, *PS1*$^{+/-}$ and *PS1*$^{-/-}$ cultures of PEFs were transiently transfected with a *HES1*–luciferase (*HES1*–luc) reporter gene in the presence or absence of exogenous Myc-tagged Notch1 (Notch1–Myc$_6$) and then cultured with control or Delta1-expressing QT6 quail cells (QT6-Delta1; Figure 2a–c). QT6-Delta1 cells activated the *HES1* promoter approximately 1.6-fold more than control QT6 cells in the absence of exogenous Notch1 (Figure 2a, bars 1,2) but activated it significantly more when Notch1–Myc$_6$ was cotransfected (Figure 2a, bars 3,4). In contrast, no effect of QT6-Delta1 cells was observed when a mutant form of the *HES1* promoter containing a deletion of the CSL binding sites, *HES1ΔAB* [14], was used (Figure 2a, bars 5–8) indicating that Delta1 cells elicit a Notch-dependent stimulation of the *HES1* promoter that is mediated by CSL binding sites. PEF cultures derived from *PS1*$^{+/-}$ and *PS1*$^{-/-}$ littermate embryos also showed ligand-dependent Notch1 activation of *HES1* that was enhanced when exogenous Notch1 was cotransfected and was dependent on the presence of CSL binding sites (Figure 2b,c). The magnitude of the ligand-induced activity was quantitatively similar in *PS1*$^{+/+}$, *PS1*$^{+/-}$ and *PS1*$^{-/-}$ cultures. Thus, the deficiency of PS1 did not significantly alter ligand-induced activation of the *HES1* promoter, indicating that PS1 is not required in this assay of Notch signaling.

Next, we studied the requirement of PS1 for Delta1-induced antagonism of MyoD by Notch1. Both Delta1 and Jagged1, two vertebrate ligands for Notch, have been shown to trigger the ability of Notch1 to inhibit myogenesis [17,18]. We found that *PS1*$^{+/+}$, *PS1*$^{+/-}$ and *PS1*$^{-/-}$ PEFs could activate *MCK*–luciferase (*MCK*–luc) approximately 2.5-fold in the presence of MyoD when cultured with

Figure 1



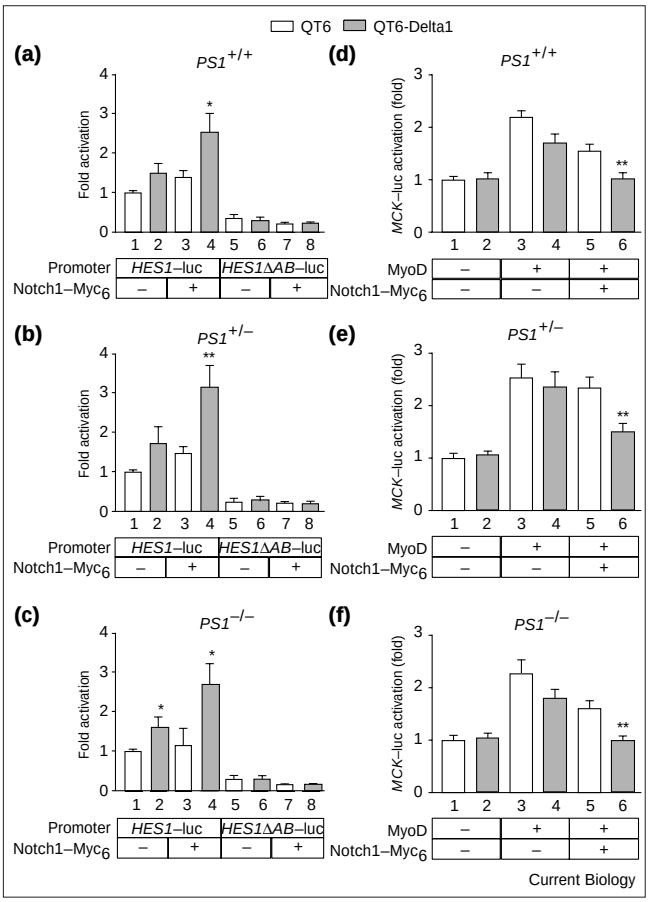
Characterization of primary embryo fibroblasts (PEFs) derived from $PS1^{+/+}$, $PS1^{+/-}$, and $PS1^{-/-}$ embryos. (a) PCR genotyping of PEFs [13] showing wild-type (WT) and mutant (mut) alleles. (b) Expression of cleaved PS1 protein in PEFs and a PS1-expressing N2a neuroblastoma line (as a positive control). (c) Expression of *PS2* mRNA, with a γ-actin control. (d) Expression of PS2 protein. (e) Expression of Notch1 protein (using the C20 antibody, Santa Cruz Biotechnologies). FL, full-length Notch1; p120, Notch1 transmembrane–intracellular domain. (f) Expression of mouse *Jagged1* (*mJag1*) mRNA with a γ-actin control. See Supplementary material for additional experimental details.

QT6 cells (Figure 2d–f, bars 1,3). We then evaluated the ability of these cells to undergo Delta1-induced inhibition of MyoD by coculturing the PEFs with Delta1-expressing QT6 cells. Whereas Delta1-QT6 cells had little effect on basal *MCK* transcription compared to control QT6 cells, Delta1-QT6 cells modestly repressed (5–20%) MyoD-activated *MCK* transcription, and significantly repressed (40–60%) *MCK* transcription in PEFs transfected with both MyoD and Notch1–Myc_6 (Fig 2d–f). Importantly, and similar to the results of *HES1* activation, the level of *MCK*–luc inhibition was the same in $PS1^{+/+}$, $PS1^{+/-}$ and $PS1^{-/-}$ cultures, demonstrating that ligand-induced inhibition of MyoD by Notch1 does not require PS1.

The intracellular domain of Notch has access to the nucleus [6–8] and a cleavage site was identified at Val1744 in this domain [6]. A fractional decline in signaling occurred when Val1744 was mutated to amino acids that diminished the cleavage of Notch1 at this site [6]. These studies support a model in which cleavage and nuclear translocation of the intracellular domain are essential for signal transduction. Recent studies imply that presenilins affect Notch signaling through a modulation of the processing of Notch similar to the function of presenilins in regulating amyloid precursor protein processing [3,4,6–8,10–12]. To observe the proteolytic release of the cleaved NICD protein, we studied wild-type and *PS1*-null PEFs transfected with a membrane-tethered intracellular domain construct of Notch1 (TM-$Notch1^{IC}$–Myc_6) and performed co-immunoprecipitation experiments with FLAG-tagged CSL^{RBP3} (CSL^{RBP3}–FLAG; Figure 3). The NICD fragment could not be detected by anti-Myc immunoblotting in either the presence or the absence of cotransfected CSL^{RBP3}–FLAG (Figure 3, lanes 1–4). Following co-immunoprecipitation using an anti-FLAG antibody, however, a protein species the size of NICD was observed in wild-type cells but not in *PS1*-null cells (Figure 3, lanes 8–9). These data confirm that the CSL^{RBP3}–NICD complex is markedly depleted in PS1-deficient cells [10].

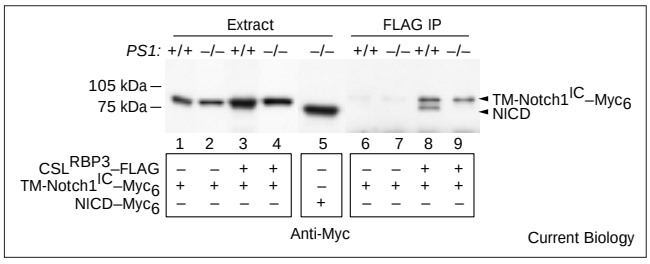
Finally, we compared hemagglutinin (HA)-tagged membrane-tethered and Myc-tagged non-membrane-tethered activated forms of Notch1, HA–TM-$Notch1^{IC}$ [19] and $Notch1^{IC}$ [20], respectively, in assays of Notch signaling in PS1-deficient and control cells (Figure 4a). HA–TM-$Notch1^{IC}$ activated the *HES1* promoter 5–7-fold in $PS1^{+/+}$ and $PS1^{+/-}$ fibroblasts. HA–TM-$Notch1^{IC}$ (Figure 4a) and TM-$Notch1^{IC}$–Myc_6 (data not shown) also activated the *HES1* promoter in PS1-deficient PEFs. Similarly, $Notch1^{IC}$ activated *HES1* in all three genotypes (Figure 4a). A slightly greater *HES1* activation was observed in *PS1*-null cells than $PS1^{+/-}$ and $PS1^{+/+}$ cells with both Notch1 constructs. We also measured the effect of PS1 deficiency on the ability of the activated Notch1 to suppress myogenesis by measuring the transactivation of an *MCK*–luc reporter gene by MyoD. PEFs were transiently transfected with *MCK*–luc along with MyoD and either HA–TM-$Notch1^{IC}$ (Figure 4b) or $Notch1^{IC}$ (Figure 4c). Transfection of MyoD reproducibly produced a 3–5-fold activation of *MCK*. HA–TM-$Notch1^{IC}$ produced 20–60% inhibition of MyoD-activated *MCK* expression over a range of DNA concentrations (Figure 4b). Similarly, the range of $Notch1^{IC}$ inhibition varied from 30% at the lowest DNA concentration to 73% at the highest (Figure 4c). The inhibition of MyoD-induced *MCK* promoter activity was equivalent in all three *PS1* genotypes. The absence of an effect of PS1 deficiency on activated Notch constructs in these two assays implies that membrane-tethered and non-membrane-tethered intracellular Notch1 constructs in mammalian cells do not (as previously observed in one study of *Drosophila* development) require presenilins for their activity [4]. Additionally, the diminution of CSL-bound NICD in *PS1*-null cells observed above did not correspond to any decline in Notch signals.

Figure 2



Delta1-dependent Notch signaling in control and *PS1*-null fibroblasts. (a–c) *HES1* activation and (d–f) MyoD antagonism were tested in (a,d) $PS1^{+/+}$, (b,e) $PS1^{+/-}$ and (c,f) $PS1^{-/-}$ cultures grown on control QT6 or Delta1-expressing QT6 cells (see Supplementary material for experimental details). (a–c) Cells were transfected with 500 ng *HES1*–luc (lanes 1–4) or 500 ng *HES1ΔAB*–luc [14] (lanes 5–8) along with Notch1–Myc_6 (1 μg) as indicated. (d–f) Cells were transfected with 500 ng *MCK*–luc (lanes 1–6) along with 500 ng the activator MyoD (lanes 3–6). The mean luciferase activation relative to lane 1 was calculated, and the significance of the level of activation with QT6 cells compared to that with QT6-Delta1 cells was assessed with a two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

Figure 3



Detection of the cleaved intracellular domain of Notch (NICD) in $PS1^{+/+}$ and $PS1^{-/-}$ fibroblasts. PEFs were transfected with TM-$Notch1^{IC}$–Myc_6 with or without CSL^{RBP3}–FLAG as indicated. Cell extracts (lanes 1–5) and immunoprecipitates produced using an anti-FLAG antibody (lanes 6–9) were immunoblotted with an anti-Myc antibody (9E10) and compared with transfected NICD–Myc_6 protein [6] (lane 5). Full-length TM-$Notch1^{IC}$–Myc_6 (arrow) alone is seen in extracts. Following immunoprecipitation, both the full length TM-$Notch1^{IC}$ and NICD fragments were immunoprecipitated from $PS1^{+/+}$ PEFs, but NICD was markedly reduced in $PS1^{-/-}$ PEFs.

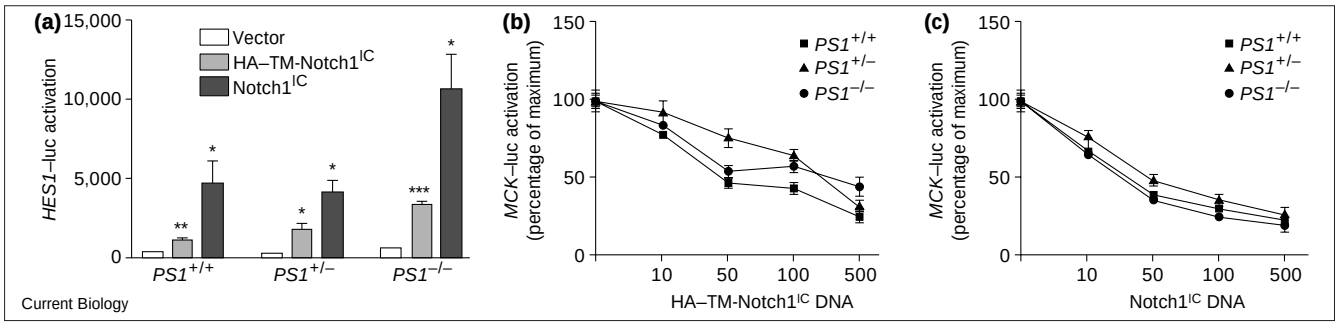
These studies reveal that a deficiency of PS1 had no measurable effect on Delta1-induced Notch1 signaling in fibroblasts, as measured by both *HES1* activation and MyoD inhibition. Notch1 signaling also persisted in *PS1*-null fibroblasts despite the virtual absence of an intracellular fragment of Notch1 (NICD) bound to CSL^{RBP3}, which has, until now, been thought to be critical for Notch signal transmission. Finally, PS1 deficiency did not impair the activity of a membrane-tethered Notch1 intracellular domain construct (HA–TM-$Notch1^{IC}$), which presumably requires cleavage for its activity. The redundancy of presenilin genes is the most likely explanation for the persistence of Notch signals in PS1-deficient cells. Similar to what was observed in *C. elegans*, mice lacking both mammalian PS homologues display a phenotype that resembles a deletion of key Notch pathway genes, more severe than a deletion of either *PS1* or *PS2* alone [5,13,21–24]. This suggests that PS2 plays a compensatory role in Notch signaling, and explains why we did not observe an impairment of ligand-induced Notch1 activation in *PS1*-null fibroblasts. In contrast, a recent study on neurite outgrowth and its control by Notch signals gives indirect evidence of impaired Notch function in *PS1*-null neurons [25]. However, we have observed no alteration of endogenous neurite growth in PS1-deficient neuron cultures, and these cultures also express PS2 (J. Palacino, B. Wolozin, B.E.B. and J.S.N., unpublished observations).

The lack of the NICD fragment in PS1-deficient cells and the preservation of Notch signaling observed here implies that production of the NICD fragment is not, as previously suggested, essential for Notch signal transduction [6,10,11]. Studies of transfected mammalian Notch1 proteins have focused on a single cleavage of the intracellular domain. In *Drosophila*, however, the endogenous intracellular domain of Notch was shown to be cleaved in several places [7,9,26] and the proteolytic products and their distribution within cells were altered in presenilin mutants [3,4]. Our data showing signaling in the virtual absence of the NICD fragment are consistent with models in which Notch signals are transduced by more than one intracellular fragment of Notch, as well as models in which an uncleaved portion of the intracellular Notch receptor activates CSL proteins, as previously proposed [9].

Supplementary material

Supplementary material including additional experimental details is available at http://current-biology.com/supmat/supmatin.htm.

Figure 4



Ligand-independent Notch signaling in $PS1^{+/+}$, $PS1^{+/-}$ and $PS1^{-/-}$ fibroblasts. (a) *HES1* activation. PEFs were transfected with *HES1*–luc (200 ng) with either vector control, HA–TM-Notch1^{IC} or Notch1^{IC}. The level of *HES1* activation by HA–TM-Notch1^{IC} and Notch1^{IC} was significantly higher than control in cells of each of the three genotypes, by a two-tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The effect of genotype was also significant by one-way analysis of variance (ANOVA; $p = 0.016$). (b,c) Inhibition of *MCK* activation. PEFs were transfected with *MCK*–luc (500 ng) along with MyoD (500 ng). In addition, increasing amounts of (b) HA–TM-Notch1^{IC} or (c) Notch1^{IC} were cotransfected. Maximal activation of *MCK*–luc is achieved in the absence of added Notch1. Percentage maximal activation of *MCK*–luc is plotted. No effect of genotype was observed by ANOVA.

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Supplementary material

Lack of requirement for Presenilin1 in Notch1 signaling

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Supplementary materials and methods

Primary fibroblasts

Embryos from *PS1* heterozygous matings were harvested into individual flasks on embryonic day E14.5 [S1]. After the head and organs were removed the body cavity was dispersed in DMEM (high glucose), 10% FBS, 2 mM L-glutamine, 1% penicillin–streptomycin (Gibco BRL). For each cell line, PCR genotyping was performed on genomic DNA extracted from corresponding organs. PCR amplification of endogenous and targeted PS1 alleles produce fragments of 500 bp and 370 bp, respectively.

Immunoblots and antibodies

Cells were extracted in SDS sample buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS) in the presence of protease inhibitors: PMSF (0.1 mM), antipain (10 μg/ml), pepstatin A (10 μg/ml), soybean trypsin inhibitor (10 μg/ml) and benzamidine (0.044%). Extracts were separated by SDS–PAGE and transferred to nitrocellulose. Polyclonal antibodies against the carboxy-terminal loop domains of human PS1 [S2] and human PS2 [S3] were used to detect PS expression using 30 μg of protein. A polyclonal antibody against the carboxy-terminal domain of mouse Notch1 (mNotch1; C20, Santa Cruz Biotechnologies) was used to detect Notch1 expression using 10 μg of protein. Myc-tagged Notch1 constructs were detected with an anti-Myc monoclonal antibody (9E10). Bound antibodies were detected with a chemiluminescent substrate (Pierce).

Northern blot hybridization

RNA was prepared from primary fibroblasts using TRIZOL Reagent (Gibco BRL). Total RNA (10 μg) was run on a 1.2% agarose gel containing 5 mM methylmercury hydroxide and was transferred to nylon by electroblotting. ^{32}P-labeled probes were prepared using the PrimeIt II kit (Stratagene). Hybridization was performed in Church buffer (1% BSA, 7% SDS, 0.5 M NaH_2PO_4 pH 7.0, 1 mM EDTA, 100 μg/ml salmon sperm DNA) at 65°C for 16 h. *Jagged1* cDNA was a gift from Genentech and *mPS2* cDNA was a gift from Luciano D'Adamio. A human γ-actin probe was used as a loading control [S4].

DNA constructs

Notch1–Myc_6 encodes mouse Notch1 (amino acids 1–2185) with a carboxy-terminal hexameric Myc tag [S5]. Notch1^{IC} contains the intracellular domain of mNotch1 with an amino terminal Myc epitope [S6]. HA–TM-Notch1^{IC} contains the transmembrane and intracellular domains of mNotch1 with an amino-terminal hemagglutinin (HA) tag and a carboxy-terminal Myc tag [S7]. TM-Notch1^{IC}–Myc_6 (also called mNotchΔE) contains the transmembrane and intracellular domains of Notch1 terminating at amino acid 2185 (at the *Xho*I site), followed by a carboxy-terminal hexameric Myc epitope [S7]. NICD–Myc_6 [S5] and MyoD were kind gifts of R. Kopan. CSL^{RBP3}–FLAG, *HES1*–luc and *HES1ΔAB*–luc were gifts from Alain Israël. *HES1*–luc contains nucleotides –194 to +160 of the *HES1* promoter cloned upstream of the luciferase gene [S8]. HES1ΔAB–luc contains a deletion of both CSL binding sites in the *HES1* promoter [S8]. *MCK*–luc contains 3.3 kb of the promoter sequence of *MCK* [S9] cloned upstream of the luciferase gene in the pGL2-basic vector (Promega).

Transfection and luciferase assays

PEFs were plated in six-well dishes at a density of 2×10^5 cells per well and transfected the following day with Lipofectamine Plus (Gibco BRL). The amount of DNA added to each well was equalized by the addition of a control plasmid, pCDNA3. Cells were harvested 48 h post transfection and luciferase activity was measured using a luminometer. To ensure equivalent transfection between lines a control reporter gene (thymidine kinase–renilla luciferase) was included with each experiment. For coculture assays [S10], QT6 or QT6-Delta1 cells (a kind gift of O. Pourquié) were plated at a density of 1×10^6 cells per well 24 h after transfection and harvested 24 h later for luciferase measurements. The data presented in Figures 2,4 represent the mean of 3–5 independent experiments, each performed in triplicate.

Immunoprecipitation

PEFs were plated at a density of 3×10^6 cells per plate in a 10 cm plate and transfected the following day. Cells were harvested 48 h after transfection in cold 0.25% n-dodecyl α-D-maltoside (Sigma) in PBS containing protease inhibitors (see *Immunoblots and antibodies*) and lysates were subjected to centrifugation at $10{,}000 \times g$ for 10 min to remove cellular debris. Lysates were precleared by the addition of purified immunoglobulin G_1 followed by incubation with protein G beads (UltraLink, Pierce). Precleared lysates were then incubated with 2 μg anti-FLAG antibody (Sigma) per 200 μg protein on ice for 45 min, and immune complexes were pulled down with protein G beads after an additional 30 min incubation at 4°C. A very small amount of uncleaved TM-Notch1^{IC} is non-specifically precipitated in the absence of cotransfected CSL^{RBP3}–FLAG (see Figure 3).

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